Br. J. Pharmacol. (1988), **94**, 199–211

Comparison of the effects of some muscarinic agonists on smooth muscle function and phosphatidylinositol turnover in the guinea-pig taenia caeci

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1 The effects of the muscarinic agonists acetylcholine (ACh), carbachol (CCh), AHR-602, and McN-A-343 on contractility and on inositol phosphate accumulation in the presence of lithium were compared in the taenia of the guinea-pig caecum.

2 Compared to CCh, ACh was a full agonist for contraction but AHR-602 and McN-A-343 were partial agonists producing 80–85% of the maximal response to CCh. Similar to previous findings with CCh, tonic contractions produced by AHR-602 and McN-A-343 were less sensitive to inhibition by nifedipine or verapamil than tonic contractions to ACh.

3 CCh and ACh produced similar increases in inositol phosphate accumulation and the effect of CCh (0.1 mM) was inhibited by atropine (IC_{50} 8.5 nM) and pirenzepine (IC_{50} 450 nM). The accumulation of inositol phosphates in the presence of AHR-602 or McN-A-343 was not significantly different ($P > 0.05$) from basal levels.

4 A concentration of 0.2 mM AHR-602 produced a parallel shift of the concentration-response curve to CCh on inositol phosphate accumulation. The IC_{50} value for inhibition of CCh (0.1 mM) was >50 fold higher than the EC_{50} value for contraction produced by the partial agonist. McN-A-343 (20 μM) produced a flattening of the concentration-response curve to CCh for inositol phosphate accumulation.

5 The results suggest that the increase in phosphatidylinositol turnover produced by muscarinic agonists, like the contractile response, involves an M_2-muscarinic receptor. AHR-602 and McN-A-343 are partial agonists for the contractile response and while producing no significant increase in phosphatidylinositol turnover inhibit the response to CCh.

Introduction

The muscarinic agonist AHR-602 (N-benzyl-3-pyrrolidyl acetate methobromide) was first shown to activate selectively muscarinic receptors on sympathetic ganglia and to have only a weak effect on many peripheral muscarinic receptors (Franko *et al.*, 1963). It does, however, readily produce contraction of the guinea-pig taenia caeci (Hobbiger *et al.*, 1969).

In many respects its activity resembles that of McN-A-343 (4-(*m*-chlorophenylcarbamoyloxy)-2-butynyl-trimethyl ammonium chloride) being 20 to 100 times less potent than the latter at ganglionic and peripheral sites (Jones, 1963; Trendelenburg, 1966; Jaramillo & Volle, 1967a,b; Fozard & Muscholl, 1972). However, differences exist between these two agonists in that AHR-602 is inactive at some receptors where McN-A-343 shows relatively high activity (Choo *et al.*, 1986) and AHR-602 functions as an agonist at some sites where McN-A-343 acts as an antagonist (Brown *et al.*, 1980).

Recently it has been demonstrated that McN-A-343 and AHR-602 are two members of a group of muscarinic agonists that have little effect on phosphatidylinositol turnover in brain (Fisher, 1986) and heart (Brown *et al.*, 1985).

In view of the suggestion that phosphatidylinositol turnover may be involved in the coupling of receptor activation to calcium mobilization and contraction of smooth muscle (Mitchell, 1982; Baron *et al.*, 1984; Sekar & Roufogalis, 1984), it was of interest to investigate the effects of these agonists on the taenia and to compare their effects, under the influence of calcium antagonists and on inositol phosphate accu-

mulation in the presence of lithium, with those of other cholinomimetics.

Methods

Measurement of [^{3}H]-inositol phosphates

Taenia caeci were dissected from guinea-pigs of either sex and washed in cold Krebs-HEPES bicarbonate (KHB) buffer (pH 7.4) containing (mM): NaCl 111, KCl 5.9, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 10 and HEPES 20. When acetylcholine (ACh) was used as the agonist the tissue was first incubated for 20 min with the irreversible anticholinesterase dyflos (DFP) (5.4 μM) in KHB buffer. After blotting and weighing, the tissue was cut into approximately 1 cm lengths before being chopped in two perpendicular planes at a thickness of 250 μm on a McIlwain tissue chopper. The chopped tissue (< 750 mg) was then washed twice with KHB buffer before being placed in 1 ml of the buffer containing 40 μCi ml^{-1} of [^{3}H]-myo-inositol to give a final concentration of 3.13 μM inositol. For larger amounts of tissue the volume was increased proportionately. Following incubation of the tissue at 37°C in a shaking water bath for 3 h, 1 ml of the buffer containing 5 mM non-radioactive myo-inositol was added and incubated for a further 10 min. The labelled tissue was then washed three times with 5 to 7 ml of KHB-inositol buffer. In experiments involving the irreversible antagonist propylbenzilylcholine mustard (PBCM) the antagonist was added to the tissue for the final 30 min of the incubation with [^{3}H]-inositol so that the washing procedure removed unbound PBCM as well as unincorporated [^{3}H]-inositol. To determine the effects of agonists on the accumulation of [^{3}H]-inositol phosphates incubations were carried out with approximately 20 mg of tissue accurately weighed into glass centrifuge tubes. Preliminary experiments established that weighing samples of wet tissue in this manner gave an over-estimate of tissue weight of $10.62 \pm 2.2\%$ ($n = 13$) compared to the initial weight of blotted tissue. No correction was made for this in the values stated in Results. Initial experiments also established that the amount of [^{3}H]-inositol phosphates accumulated by CCh (CCh) (0.3 mM) was linearly related to the amount of tissue present over the range 10–40 mg and the time of incubation over the period 5–40 min. For subsequent incubations approximately 20 mg wet weight of tissue and incubation times for agonists of 20 or 30 min duration were chosen to measure accumulation of inositol phosphates. The [^{3}H]-inositol-labelled tissue was incubated in a final volume of 0.3 ml KHB/inositol buffer containing 10 mM LiCl and other drug additions as indicated. When an antagonist was employed (apart from PBCM), the tissue was pre-incubated with the antagonist for 15 min before the addition of agonist. All experimental points were determined in duplicate or triplicate.

Incubations were terminated by addition of chloroform/methanol and the phases separated as described by Berridge *et al.* (1982). All tubes were then sonicated for 10 min to disrupt the tissue and centrifuged to separate the phases. The [^{3}H]-inositol phosphates in the aqueous phase retained on an anion exchange column were eluted with 1 ml of a solution containing 1 M ammonium formate and 0.1 M formic acid. Scintillant (10 ml ACS II, Amersham) was added to the eluate and radioactivity counted in a Tri-Carb 2000CA liquid-scintillation analyser (Packard). Results were expressed in terms of d.p.m. mg^{-1} wet weight of tissue.

During the course of this project Norris & Guth (1985) suggested that HEPES buffer may act as an antagonist at muscarinic receptors. However, in two control experiments performed with Tyrode solution, the same physiological medium used in the isolated organ bath experiments, no differences were observed in the results obtained using Tyrode solution or Krebs-HEPES bicarbonate buffer with either AHR-602 or CCh.

Separation of the inositol phosphates accumulated in the presence of CCh, performed according to the technique of Berridge *et al.* (1982) as described by Donaldson & Hill (1985), showed that the major component extracted was inositol-mono-phosphate.

Organ bath experiments

A 1 cm length of taenia caeci was isolated as described above and mounted under 0.5 g tension in a 10 ml organ bath filled with Tyrode solution containing (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, NaH_2PO_4 0.21, $MgCl_2$ 1.1, $NaHCO_3$ 12 and glucose 5.5. The solution was kept at 37°C and gassed with 95% O_2 plus 5% CO_2. Contractions were recorded using isotonic transducers (Ugo Basile 7006) connected to a Grass pen recorder (79D). Agonists remained in contact with the tissue for 4 min and a 10 min dose cycle was employed. For AHR-602, CCh and McN-A-343 the response after 4 min (tonic response) was always used for producing concentration-response curves. For ACh both the phasic and tonic responses were measured (see Results). In experiments where ACh was used as the agonist the tissue was usually pretreated for a 20 min period with the irreversible anticholinesterase dyflos (5.4 μM) as indicated. Experiments with nifedipine were carried out in a room with daylight excluded and illuminated with gold fluorescent tubes (F40T

12/Go, Sylvania) to minimize degradation. The irreversible antagonist PBCM was incubated with the tissue for a single 30 min period. All other antagonists were initially equilibrated with the tissue for 30 min with washes every 5 min and re-added after every wash. For experiments with physiological solutions containing reduced [Ca^{2+}] the tissue was preincubated in the solution for 30 min and washed every 5 min before exposure to agonists. No compensation for the change in [Ca^{2+}] was made to the Tyrode solution.

Analysis of dose-response curves

Evaluation of concentration-response relationships for agonists was carried out by fitting data by a least squares programme on a computer. The curve was based on the logistic function described by Parker & Waud (1971):

$$E = \frac{E_{max}[A]^n}{[A]^n + (EC_{50})^n}$$

where E = response, E_{max} = maximal response to the agonist, [A] = concentration of agonist, EC_{50} = concentration of agonist producing 50% of its maximal response and n = slope factor (a constant analogous to a Hill coefficient).

For estimating equipotent molar ratios, when the agonist did not produce the same maximum response as CCh, the concentration of the agonist producing the same response as that produced by the EC_{50} for CCh was ascertained.

Effects of antagonists

Where appropriate, estimates of 'pA_2' values were determined using the relationship:

$$'pA_2' = \log(DR - 1) - \log B$$

where B is the molar concentration of the antagonist and DR is the concentration-ratio (dose-ratio) produced.

Statistical comparisons between two groups of data were made by use of Student's *t* test. Simultaneous comparison of several group means (Miller, 1966) was made with a multiple comparison programme (MLTCOMP).

Drugs used

Acetylcholine chloride (Sigma), AHR-602 (N-benzyl-3-pyrrolidyl acetate methobromide) (A.H. Robins), atropine sulphate (Sigma), carbachol (Sigma), dyflos (di*iso*propyl fluorophosphate) (Sigma), lithium chloride (BDH Chemicals), McN-A-343(4-*m*-chlorophenyl carbamoyloxy-2-butynyltrimethyl-ammonium) chloride (McNeil), myo-inositol (Sigma), myo-[2-^{3}H]inositol (Amersham), nifedipine (Bayer), oxotremorine (ICN Pharmaceuticals), pirenzepine dihydrochloride (Boehringer), verapamil hydrochloride (Knoll), propylbenzilylcholine mustard (New England) (cyclized by the procedure of Burgen *et al.*, 1974).

Results

Organ bath experiments

Contractions produced by the agonists CCh, McN-A-343 and AHR-602 consisted of a single smooth tonic phase which typically reached a constant maximum within the 4 min contact period. In contrast, the response to ACh consisted of two phases, an initial fast phasic (peak) response followed by a sustained tonic response. This biphasic response to ACh was still evident in preparations pretreated with dyflos (5.4 μM for 20 min).

The EC_{50} and maximal response values (compared to CCh 3 μM) obtained for the agonists are summarized in Table 1. AHR-602 and McN-A-343 were found to be partial agonists producing 80 and 85% of the maximal response, respectively.

Contractions of the preparation produced by AHR-602 (30–300 μM, $n = 3$) were inhibited by atropine (10 μM) and were not affected by tetrodotoxin (0.3 μM) or dyflos (5.4 μM), indicating that the effect of this agonist was mediated via muscarinic receptors on the smooth muscle. Similar findings have been described for McN-A-343 (Vong & Mitchelson, 1984).

Pirenzepine (0.5 μM) produced a rightward shift of the dose-response curve to AHR-602 (3–300 μM), the calculated 'pA_2' value (see Methods) was 7.2 ± 0.2 ($n = 4$).

Effects of calcium antagonists

Nifedipine Nifedipine (3–50 nM) produced greater inhibition of the tonic phase of the response to ACh than the phasic response in dyflos-pretreated preparations (Figure 1); to produce 50% inhibition of the tonic response to ACh an approximately 10 fold lower concentration of nifedipine was required (Figure 2).

The absolute relationship between contraction and receptor occupation is not known. Therefore to compare the effect of the calcium antagonist on other cholinomimetics, control responses to these agonists were matched with tonic responses to ACh.

Nifedipine (3–50 nM) produced inhibition of responses to AHR-602 and McN-A-343 which was less than the reduction of the tonic responses to

Table 1 Comparative effects of some muscarinic agonists on the isolated taenia caeci preparation

Agonist (μM)	EC_{50} (μM) (*95% confidence limits*)	% *CCh* E_{max}* (*mean ± s.e. mean*)	*Equipotent molar ratio*	*Slope*†† (*mean ± s.e. mean*)	n
AHR-602† (6–300)	19.7 (14.2–27.3)	80.3 ± 4.4[a]		1.42 ± 0.13	16
	23.8** (15.5 – 36.6)		504		
McN-A-343 (0.6–30)	2.05 (1.55–2.72)	84.8 ± 2.3[a]		1.13 ± 0.13	15
	2.23** (1.68–2.97)		47.2		
ACh† (0.01–1)	0.06 (0.04–0.07)	96.6 ± 2.1	1.20	1.13 ± 0.10	13
CCh (0.01–3)	0.05 (0.02–0.10)	100	1.00	0.86 ± 0.13	8

* CCh E_{max} = response to carbachol (CCh) 3 μM.
† Dyflos pretreated preparations (5.4 μM, 20 min). Measurement made on phasic response for ACh.
** Concentration producing the same response as the EC_{50} for CCh.
[a] Significantly different from CCh E_{max} ($P < 0.01$, unpaired t test).
†† Slopes did not differ significantly ($P > 0.05$) from each other (MLTCOMP).

ACh. Concentrations of nifedipine required to produce 50% inhibition of the responses to AHR-602 and McN-A-343 were approximately 3.8 and 4.3 fold higher, respectively, than that required to inhibit the tonic response to ACh to the same extent (Figure 2).

Nifedipine (10 nM) sometimes converted the response to AHR-602 to two phases (Figure 3). There was an initial peak response followed by a secondary tonic phase during which the taenia underwent spontaneous activity. This activity was sometimes observed in the secondary tonic phase of ACh in these experiments (Figure 3). Responses to McN-A-343 showed a similar trend to become biphasic after nifedipine 10 nM, although the phases were not as distinct (Figure 3). Increasing the concentration of nifedipine to 50 nM left only a peak phasic response to McN-A-343 and AHR-602.

Verapamil Verapamil (0.1 μM) inhibited the tonic phase of the response to ACh significantly in both dyflos pretreated (0.1 and 1 μM, ACh) ($P < 0.05$, $n = 8$, paired t test) and untreated (1 and 10 μM) ($P < 0.01$, $n = 8$, paired t test) preparations, while the phasic portion of the response was not significantly inhibited. Phasic responses to ACh at the higher dose level were potentiated significantly by verapamil (0.1 μM) ($P < 0.05$, $n = 8$) in both these preparations but this was attributed to the drop in tone frequently caused by verapamil.

Responses to CCh, AHR-602 and McN-A-343 were reduced in the presence of verapamil (0.1 μM) but no biphasic response was evident and generally no fading of the response occurred during the 4 min contact period with the agonists. Comparison of the effect of verapamil on responses to ACh with those to CCh, AHR-602 and McN-A-343 showed that responses to the latter three agonists were always inhibited to a lesser extent than comparable tonic contractions to ACh (Table 2).

Reduction of calcium content of Tyrode solution While the tonic response to ACh was more sensitive to the effect of reduced calcium than responses to AHR-602 and McN-A-343 or the phasic response to ACh, by a factor >1.7 fold, the difference was not significant ($P > 0.05$) (Figure 4).

Analysis of the time course of the responses showed that reduced calcium altered contractions to the agonists in a different manner from nifedipine. No initial phasic response was evident with either AHR-602 or McN-A-343 and the response to either agonist developed more slowly than in normal Tyrode solution.

Phosphatidylinositol turnover

Effects of agonists CCh (3 μM–1 mM), ACh (30 μM–3 mM), and oxotremorine (0.3 μM–0.1 mM) produced a significant increase in [^{3}H]-inositol phosphate accumulation. (Table 3, Figure 5). As can be seen from the data given in Table 3, accumulation of [^{3}H]-inositol phosphates was stimulated by ACh to a similar extent to CCh whereas oxotremorine was a partial agonist in this system. Concentrations of AHR-602 (3–300 μM) and McN-A-343 (3–300 μM) produced a small increase, neither of which was significantly different from the basal value ($P > 0.05$)

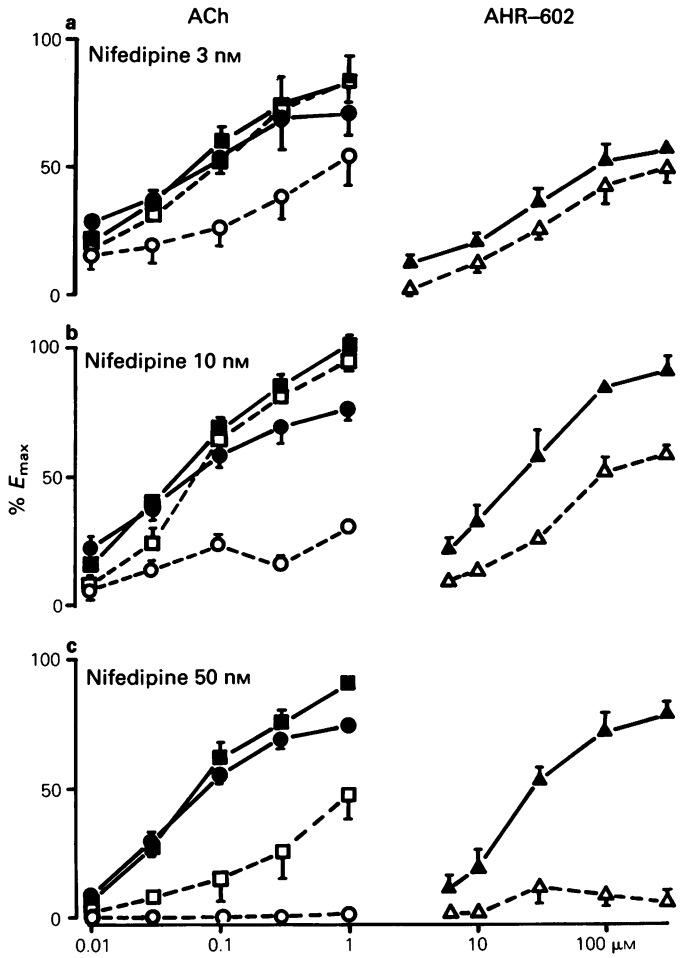

Figure 1 Effect of nifedipine (3–50 nM) on the concentration-response curves for acetylcholine (ACh) and AHR-602 in the same dyflos-pretreated preparations. For ACh both phasic (■, □) and tonic (●, ○) responses are shown. Ordinate scales: responses are expressed as a percentage of the response to a maximal dose of carbachol (CCh) (3 μM). Abscissa scale: concentration of agonist. Control curves are shown with filled symbols and continuous lines and curves in the presence of nifedipine with open symbols and broken lines. Different preparations were used for each concentration of nifedipine. For nifedipine 3 nM (a), $n = 4$; 10 nM (b), $n = 4$ and 50 nM (c), $n = 3$. Data points are shown as means, with vertical lines indicating s.e. mean unless smaller than the dimensions of the symbols.

(Figure 5 and Table 3). Raising the concentration of AHR-602 to 3 mM and McN-A-343 to 1 mM in one experiment did not produce any greater effect than 300 μM.

To determine whether CCh could increase phosphatidylinositol turnover when it produced a maximal contraction equivalent to that observed with AHR-602, some experiments were conducted after treatment of the tissue with propylbenzilylcholine mustard (PBCM). These experiments were complicated by the phenomenon previously noted by Gupta *et al.* (1976). While PBCM produced a rightward shift of the cumulative concentration-contractile response curve to CCh and a depression of the maximal contraction, some recovery of the maximum was obtained on repeated washing over 45–60 min (Table 4). The increase in phosphatidylinositol turnover produced by CCh (0.3 mM) was inhibited after treatment of the tissue with PBCM in concentrations producing inhibition of the maximal contractile response (Table 4).

Effect of muscarinic receptor antagonists Atropine (1 μM) significantly inhibited stimulation of [^{3}H]-

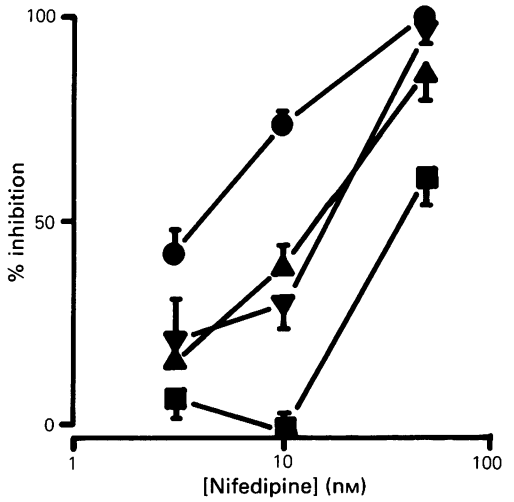

Figure 2 The percentage inhibition produced by nifedipine (3–50 nM) on the tonic (●) and phasic (■) response to acetylcholine (ACh), (0.3 μM), and the response to AHR-602 (100 μM) (▲) and McN-A-343 (10 μM) (▼). The mean control responses were similar in size, the percentage of E_{max} to carbachol (CCh) being for ACh (tonic response) 72.5 ± 2.7% of maximal response to CCh 3 μM (n = 22), ACh (phasic response) 76.9 ± 5.2% (22), McN 74.4 ± 3.4% (11) and AHR 69.8 ± 5.3% (11). Each point represents the mean and vertical lines indicate s.e. mean.

inositol phosphate accumulation due to CCh (0.1 mM, n = 3), ACh (3 mM, n = 5) and oxotremorine (0.1 mM, n = 4) (paired t test, $P < 0.05$) (Table 5).

Atropine in a concentration of 3 μM did not fully inhibit the responses to CCh (0.1 mM) (Figure 6). A similar finding was made with ACh (0.3 mM) (plus dyflos) the maximum inhibition being 73.7 ± 9.1% (4). This finding was not due to an action of the agonists on nicotinic receptors as hexamethonium (0.1 mM) did not have any significant effect on the increase in phosphatidylinositol produced by CCh (0.1 mM) (102.4 ± 25.4% (4) of control).

Pirenzepine (10 nM–30 μM) and atropine (0.3 nM–3 μM) produced dose-dependent inhibition of CCh-stimulated accumulation of [^{3}H]-inositol phosphates (Figure 6). The IC_{50} values obtained for atropine 8.50 nM (1.15–62.7 nM, n = 5) and pirenzepine 450 nM (40.1 nM–5.04 μM, n = 5) displayed a difference of approximately 53 fold.

Effects of AHR-602 and McN-A-343 on phosphatidylinositol responses to CCh AHR-602 at a concentration of 0.2 mM, approximately 10 fold higher than the EC_{50} for contraction, produced a 2.2 fold shift in the concentration-phosphatidylinositol response curve for CCh providing a calculated pA_2 value of 3.8 (Figure 7).

Raising the concentration of AHR-602 to 1 mM produced only 32.9 ± 5.0% (9) inhibition of the response to CCh (0.1 mM).

McN-A-343 (209 μM) produced some flattening of the concentration-response curve to CCh (Figure 7). A concentration of 1 mM McN-A-343 produced 66.3 ± 8.5% (5) inhibition of the response to CCh (0.1 mM).

Effect of verapamil Verapamil (0.1 μM) did not significantly ($P > 0.05$) affect the accumulation of [^{3}H]-inositol phosphates stimulated by ACh (30 μM–3 mM), although this concentration of verapamil was capable of significantly reducing the tonic contractile response to ACh (0.1 and 1 μM) in functional studies (see above). For example the percentage increase in [^{3}H]-inositol phosphates produced by ACh 3 mM in the absence and presence of verapa-

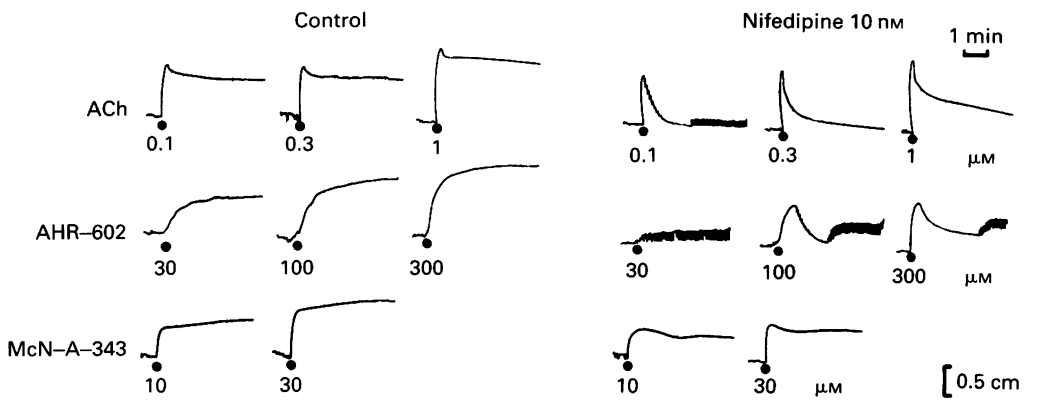

Figure 3 The effect of nifedipine (10 nM) on responses to acetylcholine (ACh), AHR-602 and McN-A-343 in dyflos-pretreated taenia. Contractions were recorded isotonically.

Table 2 Effect of verapamil (0.1 μM) on tonic responses to acetylcholine (ACh) and carbachol (CCh), AHR-602 and McN-A-343

Treatment	n	*Agonist*	*Conc.* (μM)	% *CCh* E_{max}*	*% inhibition by verapamil* (0.1 μM)	P**
Untreated	8	ACh	1	51.8 ± 2.3	61.6 ± 4.7	
		CCh	0.03	44.3 ± 2.0	33.5 ± 7.9	<0.01
Untreated	6	ACh	0.3	44.0 ± 6.2	74.2 ± 4.9	
		AHR-602	10	44.6 ± 2.0	54.8 ± 7.4	<0.05
Untreated	6	ACh	1	50.5 ± 5.3	59.6 ± 10.8	
		McN-A-343	3	47.9 ± 5.7	35.5 ± 9.0	<0.05
Dyflos pretreated	12	ACh	0.1	51.7 ± 3.5	48.7 ± 8.4	
		CCh	0.1	58.5 ± 2.8	13.9 ± 6.3	<0.01

Results shown are means ± s.e. mean.
* See Table 1.
** Comparison with corresponding response to acetylcholine after verapamil.

Table 3 Effects of some muscarinic agonists on [^{3}H]-inositol phosphate accumulation in the guinea-pig taenia caeci over 20 or 30 min as indicated

Agonist	EC_{50} (μM) (*95% confidence limits*)	(n)	*Factor of stimulation*† *20 min* (n)	*30 min* (n)	% *CCh* E_{max}*	*Slope* (*mean ± s.e. mean*)
ACh[a]	108 (37.2–315)	6	4.04 ± 0.69*** (6)		87.6	0.72 ± 0.12
CCh	27.8 (18.1–42.7)	6	4.47 ± 0.39*** (11)	5.72 ± 0.70*** (8)	100	1.18 ± 0.14
Oxotremorine	6.65 (0.90–49.2)	3		2.79 ± 0.50** (4)	37.9	1.03 ± 0.21
McN-A-343	–††	4		1.62 ± 0.20 (4)	13.1	–
AHR-602	–††	6		1.27 ± 0.13 (6)	5.7	–

[a] Dyflos present (5.4 μM).
† The maximum response of the agonist (acetylcholine (ACh) 3 mM, carbachol (CCh) 0.3 mM, oxotremorine 0.1 mM, McN-A-343 0.3 mM, AHR-602 0.3 mM) divided by the basal value for each experiment (factor = 1 for no stimulation).
* % CCh E_{max} is obtained by dividing the maximum response of the agonists (factor of stimulation − 1) by CCh response (factor of stimulation − 1) listed above.
†† Response too small to determine EC_{50}.
** Significantly different from 1 ($P < 0.05$, paired t test).
*** Significantly different from 1 ($P < 0.01$, paired t test).

Table 4 Effect of propylbenzilylcholine mustard (PBCM) for 30 min on maximal contractile response to carbachol (CCh) and on the phosphoinositide (PI) response to CCh (0.3 mM)

[*PBCM*] (μM)	% *CCh maximal contraction*† *Initial*	*Subsequent* (n)	*PI response to CCh* (0.3 mM) % *control* (n)
0.2	–	–	78.3 ± 8.9 (7)
1.0	77.7 ± 5.3	93.3 ± 3.0 (4)	38.0 ± 7.2 (6)
3.0	65.0 ± 7.9	91.1 ± 1.6 (4)	29.1 ± 11.4 (4)
10.0	39.8 ± 5.6	64.2 ± 3.0 (4)	11.4 ± 4.1 (4)

† Tetrodotoxin (0.3 μM) present throughout; cumulative concentration-response curves. 'Initial' maximal response (CCh 0.2 mM) determined after washing tissue twice at end of a 30 min incubation period. 'Subsequent' maximal response determined after repeated washing, 45–60 min following end of incubation with PBCM.

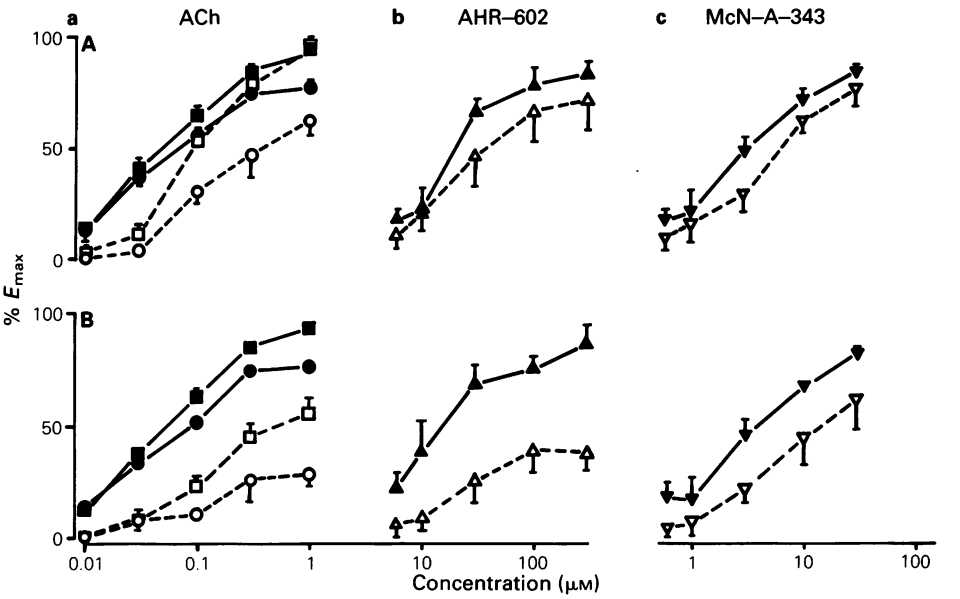

Figure 4 The effect of reducing the calcium content of Tyrode solution to 1/10 (A) and 1/30 (B) normal calcium on concentration-response curves for (a) acetylcholine (ACh) (phasic response ■, □; tonic response ●, ○), (b) AHR-602 and (c) McN-A-343 in dyflos-treated preparations. For ACh and AHR-602 data were obtained from the same preparations in 1/10 Ca^{2+} ($n = 4$) and 1/30 Ca^{2+} ($n = 4$). For McN-A-343 similar comparisons were made with 1/10 Ca^{2+} ($n = 4$) and 1/30 Ca^{2+} ($n = 3$) although data for ACh are not shown. Other details as in Figure 1.

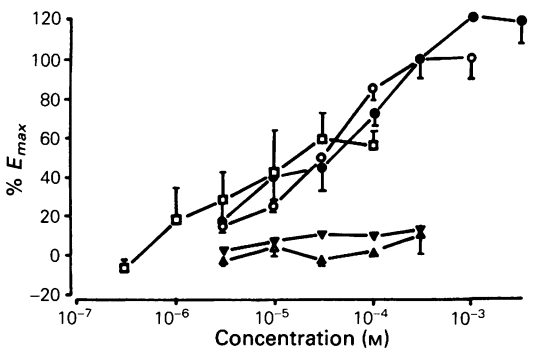

Figure 5 Concentration-response curves of muscarinic agonists on [^{3}H]-inositol phosphate accumulation in the presence of Li^+. The figure incorporates only those experiments listed in Table 3 where responses to agonists could be expressed as a percentage of that to carbachol (CCh, 0.3 mM) in the same experiment. For CCh (○) $n = 6$, except for 1 mM where $n = 3$, oxotremorine (□) $n = 3$, except for 30 μM and 0.1 mM where $n = 4$, McN-A-343 (▼) $n = 2$, AHR-602 (▲) $n = 3$ and acetylcholine in the presence of dyflos (5.4 μM) (●) $n = 6$. Note that ACh responses are expressed as a percentage of the response to CCh (0.3 mM) in the presence of dyflos. These values for CCh are not listed in Table 3. Each point represents the mean and vertical lines indicate s.e. mean.

mil (0.1 μM) was 492 ± 112 ($n = 3$) and 499 ± 107 ($n = 3$) respectively. Also, verapamil (0.3 μM or 1.0 μM) did not significantly ($P > 0.05$) inhibit stimulation due to CCh (0.3 mM) (paired t test, $n = 3$ and 4, respectively), although verapamil (1.0 μM) produced 78.2 ± 8.8% ($n = 3$) inhibition of the maximum contractile response to CCh (3 μM) in isolated tissue experiments.

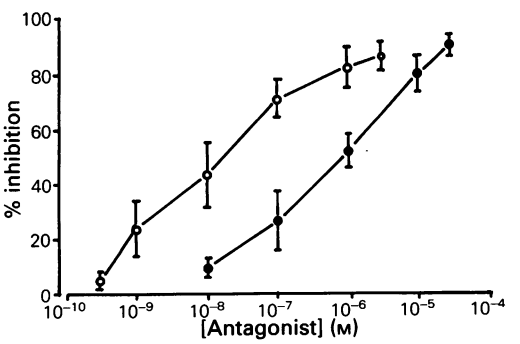

Figure 6 Inhibition of carbachol (0.1 mM)-stimulated [^{3}H]-inositol phosphate accumulation by atropine (0.3 nM–3 μM) ($n = 5$) (○) and pirenzepine (10 nM–30 μM) ($n = 5$) (●). Each point represents the mean and vertical lines indicate s.e. mean.

Table 5 Effect of atropine 1 μM on [^{3}H]-inositol phosphate accumulation stimulated by some muscarinic agonists

Agonist (mM)	n	*Factor of stimulation** (*mean* ± *s.e. mean*)	*Factor of stimulation after atropine* 1 μM (*mean* ± *s.e. mean*)	P**
CCh (0.1)	3	4.11 ± 0.47	1.56 ± 0.17	<0.01
ACh (3)	5	4.76 ± 0.63	2.56 ± 0.48	<0.05
Oxotremorine (0.1)	4	2.79 ± 0.50	1.74 ± 0.37	<0.01
McN-A-343 (0.3)	4	1.62 ± 0.20	1.34 ± 0.26	>0.05
AHR-602 (0.1)	4	1.27 ± 0.13	1.20 ± 0.08	>0.05

* See Table 3.
** Paired *t* test.

Discussion

While AHR-602 is generally considered to be a stimulant of muscarinic receptors on ganglion cells (Franko *et al.*, 1963), in the taenia both AHR-602 and CCh produced a contraction of smooth muscle that was due to activation of peripheral muscarinic receptors on the smooth muscle. Responses to either were unaffected by tetrodotoxin or dyflos pretreatment, indicating no indirect mechanisms for the agonists. Previous work has established that the other agonists investigated, ACh and McN-A-343 also act directly on the smooth muscle (Vong & Mitchelson, 1984). The EC_{50} values, maximal response relative to CCh and slope values are similar to those found previously. AHR-602 like McN-A-343 produced a tonic response and was a partial agonist, producing a response of approximately 80% of the maximum.

In experiments to compare effects of calcium antagonists, matched responses to all agonists were obtained as Cauvin *et al.* (1982) have indicated that it is necessary to use equi-effective concentrations of agonists when attempting to detect differences in sensitivity to calcium entry blocking drugs. The effect of verapamil or nifedipine on responses to ACh was to produce selective inhibition of the tonic phase of the response. The tonic phase of the response to CCh was more resistant to inhibition by verapamil. Similar findings have been made with gallopamil and nifedipine (Mitchelson & Ziegler, 1984). The tonic responses to McN-A-343 and AHR-602 were inhibited by verapamil and nifedipine but also to a lesser extent than ACh; for nifedipine a four fold higher concentration of the calcium antagonist was required to produce 50% inhibition of the tonic response to the partial agonists. The concentration range of nifedipine was comparable to that required to inhibit contractions of taenia induced by calcium (Spedding, 1982; Armstrong & Lefournier, 1985) and the uptake of $^{45}Ca^{2+}$ induced by the cholinomimetic *cis*-dioxolane in ileum (Rosenberger *et al.*, 1979). Although verapamil may interact allosterically with [^{3}H]-N-methylhyoscine at muscarinic receptors (Baumgold, 1986) and verapamil and nifedipine can displace [^{3}H]-quinuclidinyl benzilate from binding sites (Cavey *et al.*, 1977; Jim *et al.*, 1981; Thayer *et al.*, 1985), the concentrations required are approximately 100 and 500 fold higher, respectively, than those used in the present experiments and thus interaction between calcium antagonists and the agonists was unlikely to be at the muscarinic receptor. Reduction in the external calcium concentration also maintained the trend for the tonic phase of the response to ACh to be more susceptible to inhibition than either AHR-602 or McN-A-343.

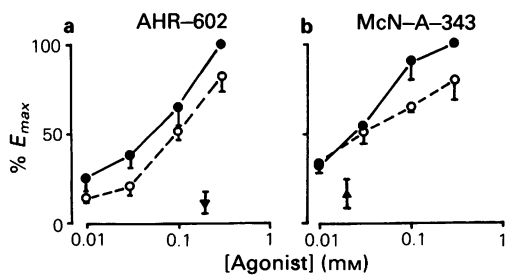

Figure 7 The effect of carbachol on [^{3}H]-inositol phosphate accumulation in the absence (●) and presence of a partial agonist (○). (a) Shows the effect of AHR-602 (0.2 mM) alone (▼) ($n = 5$) and on the response to carbachol (○) and (b) shows the effect of McN-A-343 (20 μM) alone (▲) $n = 3$) and on the response to carbachol (○). Each point represents the mean and vertical lines indicate s.e. mean.

These findings suggest that activation of the muscarinic receptor by AHR-602 or McN-A-343 is different from that by ACh. If the agonists activated the receptor in an identical manner coupling of receptor activation to response should be identical and equally susceptible to the calcium antagonists or reduction in the external calcium concentration.

A current explanation of smooth muscle contraction suggests that extracellular Ca^{2+} entry occurs secondary to the emptying of an intracellular Ca^{2+}

store by inositol triphosphate (IP_3). This is followed by Ca^{2+} entry into the cell through Ca^{2+} channels possibly activated by inositol 1,3,4,5 tetra*kis*phosphate (IP_4) (Taylor, 1987). Thus the entry of Ca^{2+} is not directly receptor-linked but occurs via an intermediate. Also, different proteins and sources of calcium appear to be utilized for phasic and tonic responses by agonists (Bitar *et al.*, 1986; Howe *et al.*, 1986; Park & Rasmussen, 1987). For example in rabbit iris, phasic responses to CCh involve IP_3 production and myosin light chain phosphorylation, whereas extracellular Ca^{2+} is required for the sustained phosphorylation of myosin light chain associated with the tonic phase of the contraction (Howe *et al.*, 1986). Thus in taenia the phasic response to ACh may involve an intracellular source of Ca^{2+} whereas the tonic phase may involve an extracellular source of Ca^{2+} requiring the opening of Ca^{2+} channels.

The reason for the variable sensitivity of the tonic phases of the response to various agonists is not clear. Brading & Sneddon (1980) have shown that CCh can utilize several sources of Ca^{2+} for the contractile response in taenia. At this stage it is not known if different subtypes of receptors or different coupling mechanisms are involved such as those Han *et al.* (1987) have recently suggested for α-adrenoceptor agonists. It is conceivable that muscarinic agonists may show different sensitivities for mobilizing various sources of calcium and this may explain the varying sensitivities of agonists towards calcium antagonists and reduced concentrations of calcium. Also it is possible that different agonists activate different phosphoinositide pathways as suggested by Gurwitz & Sokolovsky (1987). These workers found the increase in phosphoinositide hydrolysis produced by oxotremorine or CCh could be differentiated by tetrodotoxin in heart or brain.

The accumulation of [^{3}H]-inositol phosphates by the taenia was enhanced up to 6 fold by CCh over 20 to 30 min and this effect was inhibited by atropine. Oxotremorine was only a weak activator of the system being a partial agonist, while McN-A-343 and AHR-602 were the least active. This finding is in accordance with previous studies in guinea-pig cerebral cortex (Fisher, 1986; Fisher *et al.*, 1983), astrocytoma 1321 N1 cells (Brown *et al.*, 1985) and chick embryo heart (Brown *et al.*, 1985).

The most likely explanation of the findings is that contraction by a full agonist involves activation of only a small fraction of receptors whereas the phosphatidylinositol response has little or no receptor reserve. Support for this concept is provided by the large difference between the EC_{50} for contraction produced by CCh and the EC_{50} for inositol phosphate accumulation. Partial agonists on the other hand must activate the receptors fully for maximal contraction and inositol phosphate accumulation. However, this may involve only a small fraction of the available capacity for phosphoinositide hydrolysis. To answer this point experiments were conducted to determine whether CCh would increase phosphoinositide hydrolysis after irreversible block of spare receptors with PBCM. One problem with PBCM is that only the shift of the concentration-response curve for contraction in ileal smooth muscle appears irreversible, suppression of the maximal response recovers to some extent on repeated washing (Gupta *et al.*, 1976; Siegel & Triggle, 1982). This is possibly due to some limited hydrolysis of the PBCM-receptor bond, although other explanations have been offered (Gupta *et al.*, 1976). In the taenia while almost full recovery of the maximal response was found to occur on repeated washing when $\leqslant 3\,\mu$M PBCM was used, phosphoinositide hydrolysis was only approximately 30% of control. The findings with oxotremorine showed reasonable agreement with these data. While a full agonist for contraction (Grana *et al.*, 1986), oxotremorine produced only 30% of the inositol phosphate accumulation observed with CCh. With a higher concentration of PBCM ($10\,\mu$M) the maximal response recovered to 64% control with repeated washing and phosphoinositide hydrolysis was only approximately 10% of control. These results require considerable caution in interpretation since the degree of receptor inactivation in the phosphatidylinositol experiments was not directly determined and Fewtrell & Rang (1973) showed that receptor alkylation by PBCM has a slower time course in muscle strips than does inhibition of the contractile response. Nevertheless the findings for CCh after PBCM ($10\,\mu$M) treatment are comparable with the partial agonists McN-A-343 and AHR-602 being able to produce up to 80% maximal contraction without producing significant increases in inositol phosphates. Furthermore, the two partial agonists produced some inhibition of the phosphoinositide response to CCh, suggesting that they act on the receptor involved in the phosphoinositide response. In the central nervous system Fisher *et al.* (1983) have shown that the partial agonist oxotremorine while producing little stimulation of phosphoinositide hydrolysis, inhibited the stimulation produced by CCh in a similar concentration range. Also, Gil & Wolfe (1985) have demonstrated a similar interaction between oxotremorine and McN-A-343 in parotid gland where oxotremorine is a full agonist.

One finding, however, is not in agreement with this explanation. While both AHR-602 and McN-A-343 produced some inhibition of the response to CCh, the shift produced by AHR-602 ($200\,\mu$M) was

approximately 10 fold less than would be predicted on the basis of the ability of the partial agonist to contract the tissue. Increasing the concentration up to 1 mM still produced <50% inhibition of the response to CCh (0.1 mM). The reason for these findings are not clear. The results of Gurwitz & Sokolovsky (1987) referred to above could explain the results if CCh were able to activate two pathways for inositol phosphate production only one of which was inhibited by AHR-602. Further work is needed to settle this point.

The presence of verapamil in concentrations sufficient to inhibit contractions to ACh or CCh did not modify the response of these agonists on phospholipids, indicating that the action of the calcium antagonist is exerted at some point in the excitation-contraction coupling process distal to the stimulation of phosphoinositide hydrolysis. It cannot be argued that the persistence of the phasic response to ACh in the presence of the calcium antagonist could account for the maintained increase in inositol phosphate accumulation, as the phasic response waned rapidly whereas inositol phosphate accumulation continued throughout the incubation period. Similar findings have been obtained in guinea-pig ileal longitudinal muscle with gallopamil and CCh (Best *et al.*, 1985).

Pirenzepine and atropine inhibited the effects of CCh on phosphoinositide hydrolysis. The relative potency of atropine and pirenzepine (approximately 50 fold difference) suggested that the receptor is of the 'M_2' variety. Contraction of the taenia involves an M_2-receptor for McN-A-343 and acetylcholine (Vong & Mitchelson, 1984) with pA_2 values of 6.9 and this also appeared to be the case for AHR-602, as a 'pA_2' value of 7.2 for pirenzepine was obtained with this agonist. Atropine was found to have a pA_2 value of 9.0 for contractions with either ACh or McN-A-343. Turnover of phosphatidylinositol has been linked to both M_1- and M_2-receptors in the brain (Smith & Yamamura, 1985; Gil & Wolfe, 1985; Fisher, 1986) and to M_2-receptors in peripheral tissues such as the chick ventricle (Brown *et al.*, 1985) and in astrocytoma 1321 N1 cells (Brown *et al.*, 1985). In guinea-pig heart (Woodcock *et al.*, 1987) and in guinea-pig myometrium (Leiber *et al.*, 1986) phosphatidylinositol turnover has been linked to an M_1 site showing high affinity for pirenzepine.

Thus, in conclusion, AHR-602 and McN-A-343 activate muscarinic receptors on smooth muscle to cause contraction but are partial agonists compared to CCh or ACh. The tonic response of these agonists may be differentiated from that of ACh by their relatively greater resistance to calcium antagonists and from both ACh and CCh by their failure to produce a significant increase in inositol phosphates. However, they inhibited the effect of CCh on inositol phosphate accumulation, indicating an interaction at the site of action of CCh. Atropine or pirenzepine but not hexamethonium or verapamil inhibited the effect of CCh on phosphatidylinositol. The relative potency of the two muscarinic receptor antagonists suggests involvement of an M_2-muscarinic receptor in the response similar to that involved in contraction.

This work was supported by a grant from the National Health and Medical Research Council of Australia. Technical assistance by P. Napier, S. Darroch and assistance in computing by M. Quinn are greatly appreciated. Gifts of drugs from the following are gratefully acknowledged: A.H. Robins (AHR-602), McNeil (McN-A-343), Bayer (nifedipine), Boehringer Ingelheim (pirenzepine), Knoll (verapamil).

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(Received August 4, 1987
Revised November 30, 1987
Accepted December 7, 1987)